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Evidence for Interspecies Gene Transfer in the Evolution of 2,4-Dichlorophenoxyacetic Acid Degraders

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Small-subunit ribosomal DNA (SSU rDNA) from 20 phenotypically distinct strains of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria was partially sequenced, yielding 18 unique strains belonging to members of the alpha, beta, and gamma subgroups of the class *Proteobacteria*. To understand the origin of 2,4-D degradation in this diverse collection, the first gene in the 2,4-D pathway, *tfdA*, was sequenced. The sequences fell into three unique classes found in various members of the beta and gamma subgroups of *Proteobacteria*. None of the α-Proteobacteria yielded *tfdA* PCR products. A comparison of the dendrogram of the *tfdA* genes with that of the SSU rDNA genes demonstrated incongruency in phylogenies, and hence 2,4-D degradation must have originated from gene transfer between species. Only those strains with *tfdA* sequences highly similar to the *tfdA* sequence of strain JMP134 (*tfdA* class I) transferred all the 2,4-D genes and conferred the 2,4-D degradation phenotype to a *Burkholderia cepacia* recipient.

Bacteria capable of mineralizing 2,4-dichlorophenoxyacetic acid (2,4-D), a commonly used herbicide, are found in many different phylogenetic groups (2, 3, 7, 11, 22, 23). Evidence suggests that numerous variants of 2,4-D catabolic genes exist and that catabolic operons consist of a near-random mixing of these variants (7). Interspecies gene transfer is a well-documented phenomenon (13), and horizontal gene transfer of the 2,4-D-degrading plasmid pJP4 has been shown (3, 5). However, not all 2,4-D catabolic operons are found on plasmids (10, 11, 16, 20). The extent to which other 2,4-D genes have been exchanged in nature is unknown. The aim of this research was to assess the role of horizontal gene transfer in the evolution of 2,4-D-degrading strains. This article summarizes the results of two aspects of this work—the study of the transfer of the entire 2,4-D pathway by using standard mating experiments and a phylogenetic study of the *tfdA* gene. The *tfdA* gene codes for an α-ketoglutarate-dependent 2,4-D dioxygenase which converts 2,4-D into 2,4-dichlorophenol and glyoxylate (6). This 861-bp gene was first sequenced from *Ralstonia eutropha* JMP134 (19). Two more *tfdA* genes were cloned from chromosomal locations in *Burkholderia* strain RASC and *Burkholderia* strain TFD6 (16, 20). These proved to be identical to each other and 78.5% similar to the original. An alignment of the two variants allowed conserved areas to be identified and primers to be designed for the amplification of *tfdA*-like genes from other sources (24). Sequence analysis of putative *tfdA* fragments and the small-subunit ribosomal DNA (SSU rDNA) of the strains carrying them allowed us to construct phylogenies of the genes and their hosts and to look for congruency between them.

Mating experiments. A collection of 2,4-D degraders containing 15 unique strains as determined by genomic fingerprinting (7) was used as a source of donors in a series of mating experiments (Table 1). *Burkholderia cepacia* D5, lacking the ability to grow on 2,4-D and not hybridizing to any *tfd* genes, was used as a recipient in mating experiments. Strain D5 contains neomycin phosphotransferase genes (*nptII*) carried on transposon Tn*5* and is resistant to 50 μg each of kanamycin, carbenicillin, and bacitracin per ml. All of the 2,4-D strains used were sensitive to these antibiotics. Filter matings were performed with a donor-to-recipient ratio of 1:10. Colonies which grew on selective medium (500 ppm of 2,4-D in mineral salts agar [MMO] [23] including 50 μg of kanamycin, carbenicillin, and bacitracin per ml) were subjected to further tests. Their ability to catabolize 2,4-D was tested in liquid medium (same composition as that described above).

The disappearance of 2,4-D from the culture medium was monitored by high-performance liquid chromatography. Cells were removed by centrifugation, and the supernatant was filtered through 0.2-μm-pore-size filters. These samples were then analyzed on a Lichrosorb Rp-18 column (Anspec Co., Ann Arbor, Mich.) with 60% methanol–40% 0.1% H_3PO_4 as the eluant. 2,4-D was detected by measuring light absorption at 230 nm. The presence of *tfd* genes was detected by hybridizing colony blots with a DNA probe derived from the entire pJP4 plasmid. The identity of the colonies was confirmed by probing with the *nptII* gene of Tn*5* (found in *B. cepacia* D5). Probes were labeled with random hexanucleotides incorporating [^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Boston, Mass.). Hybridizations were done under high-stringency conditions by using 50% formamide and Denhardt's solution (18) at 42°C. Of the 15 unique strains tested, 9 transferred 2,4-D degradation abilities to D5. This transfer was confirmed by hybridization with pJP4 for eight of these strains. *B. cepacia* RASC could transfer degradative abilities, but neither it nor the transconjugant hybridized to the pJP4 probe. Work subsequent to this study has confirmed that the genes carried by RASC do not hybridize to those found on pJP4 under high-stringency conditions (7).

Phylogenetic analyses. Total genomic DNA was isolated from 20 unique 2,4-D-degrading strains (including all 15 used for mating experiments) grown on 500 ppm of 2,4-D mineral salts medium amended with 50 ppm of yeast extract. SSU rDNA was amplified by using fD1 and rD1 as primers (25). Putative *tfdA* fragments were amplified by using primers TVU and TVL as previously described (24). PCR products were purified with a Gene Clean kit (Bio 101, La Jolla, Calif.). Sequencing was done with an Applied Biosystems model 373A

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TABLE 1. 2,4-D-degrading strains, geographic origins, and GenBank accession numbers

Strain	GenBank accession no. (SSU rDNA)	Origin	Most similar to genus and/or species[a]	Transfer[b]	*tfdA* type[c]	GenBank accession no. (*tfdA* gene)	Reference or source
JMP134	AF049542	Australia	*Ralstonia eutropha*	+	I	M16730	3
EML1549	AF049546	Oregon	*Burkholderia* sp.	+	I		2
TFD39	AF049539	Saskatchewan	*Burkholderia* sp.	+	I	U43197	23
K712	AF049543	Michigan	*Burkholderia* sp.	+	I	U43276	11
TFD9	AF049537	Saskatchewan	*Alcaligenes xylosoxidans*	+	I	U43276	23
TFD41	AF049541	Michigan	*Ralstonia eutropha*	+	I		23
TFD38	AF049540	Michigan	*Ralstonia eutropha*	+	ND[c]		23
TFD23	AF049536	Michigan	*Rhodoferax fermentans*	+	I	U43276	23
RASC	AF049544	Oregon	*Burkholderia* sp.	(+)	II	U25717	2
TFD6	AF049546	Michigan	*Burkholderia* sp.	−	II		23
TFD2	AF049545	Michigan	*Burkholderia* sp.	−	II		23
TFD31	AF049536	Saskatchewan	*Rhodoferax fermentans*	−	III		23
B6-9	AF049538	Ontario	*Rhodoferax fermentans*	ND	III	U43196	9
I-18	U22836	Oregon	*Halomonas* sp.	ND	III	U22499	15
K1443	AF049531	Michigan	*Sphingomonas* sp.	−		—[d]	11
2,4-D1	AF049535	Montana	*Sphingomonas* sp.	−		—	R. Sanford
B6-5	AF049533	Ontario	*Sphingomonas* sp.	ND		—	9
B6-10	AF049534	Ontario	*Sphingomonas* sp.	ND		—	9
EML146	AF049532	Oregon	*Sphingomonas* sp.	−		—	2
M1	AF049530	French Polynesia	*Rhodospeudomonas* sp.	ND			R. Fulthorpe

[a] The generus and/or species most similar to the strain is given based on similarities of SSU rDNA sequences.
[b] Symbols: +, able to transfer 2,4-D degradation to *B. cepacia* D5; (+), able to transfer at very low frequency; −, no transfer detected.
[c] ND, not determined.
[d] —, no amplificate was obtained.

automatic sequencer (Perkin-Elmer Cetus) by using fluorescently labeled dye termination at the Michigan State University Sequencing Facility. The sequencing primer used for SSU rDNA fragments was 519R (5′ GTA TTA CCG CGG CTG CTG G-3′). For *tfdA* fragments, the sequencing primers were the same as the amplification primers. GenBank accession numbers for these sequences are given in Table 1.

The SSU rDNA sequences were compared to sequences in GenBank by using the Basic Local Alignment Search Tool (BLAST) (1), and those strains with the highest maximal segment

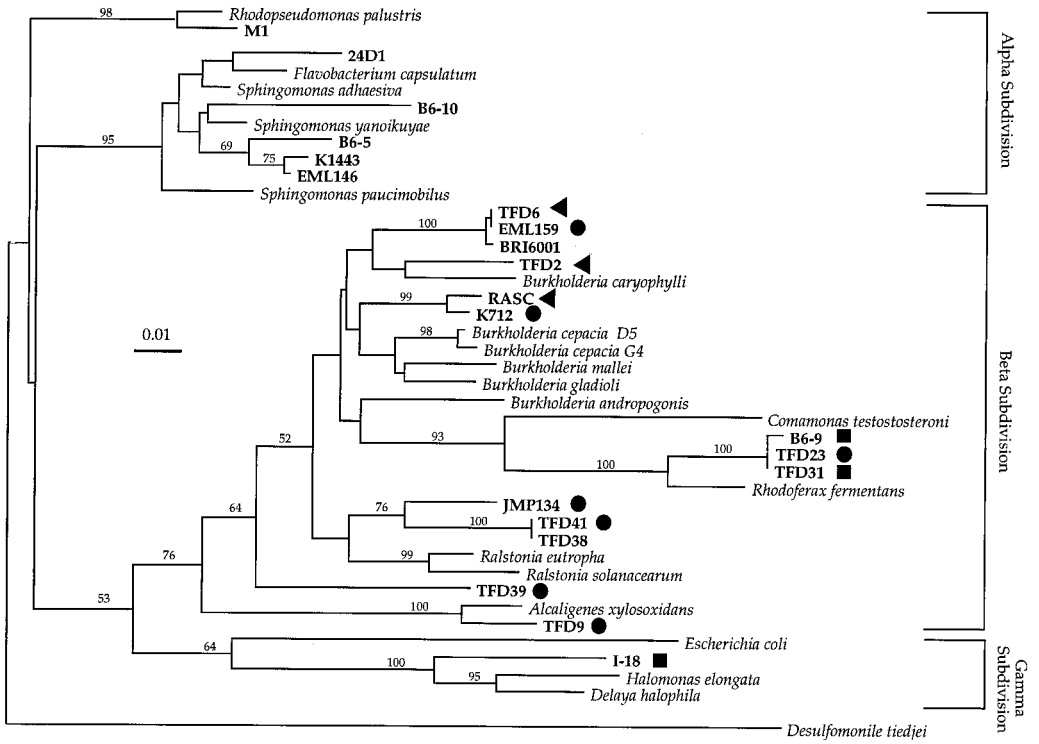

FIG. 1. Neighbor-joining dendrogram (Jukes-Cantor distances) of SSU rDNA from 2,4-D-degrading bacteria (indicated in boldface type) and reference strains (indicated in italic type). Class I (●), class II (▲), and class III (■) types of *tfdA* genes are indicated. Bootstrap confidence limits (percentages) are indicated above each branch. Scale bar represents a Jukes-Cantor distance of 0.01.

pair scores were retrieved from GenBank and included in the phylogenetic analysis. Sequences were aligned manually with the software SeqEd (Applied Biosystems) and with MacClade (14). Sites where nucleotides were not resolved for all sequences were deleted from the alignment, as were those nucleotides corresponding to the small loop in this region that is absent in the alpha subgroup of the class *Proteobacteria*. These deletions left 283 unambiguous sites for the construction of the SSU rDNA phylogenies. Phylogenetic trees were constructed by using the neighbor-joining analysis of pairwise Jukes-Cantor distances (4), and the topology was confirmed by using the maximum parsimony method PAUP (21). *Desulfomonile tiedjei* of the δ-Proteobacteria was used as an outgroup. Bootstrap analysis based on 100 replicates was used to place confidence estimates on the tree. Only bootstrap values of greater than 50 were used.

2,4-D degrader diversity. The 2,4-D degraders in this study were distributed throughout the alpha, beta, and gamma subgroups of the *Proteobacteria* (Fig. 1). The lack of representation of gram-positive bacteria is likely a reflection of isolation methods, not of the lack of gram-positive 2,4-D degraders. The majority of these strains were members of the beta subgroup of *Proteobacteria*, five of which were most closely related to the genus *Burkholderia*, having at least 92% sequence similarity with each other. Three were closely related to *Rhodoferax fermentans* (close to the class *Comamonadaceae*), three were related to *Ralstonia eutropha*, and one was related to *Alcaligenes xylosoxidans*. TFD39 falls outside any clear cluster. One member of the γ-Proteobacteria, strain I-18, a haloalkaliphile, was found to be closely related to the salt-loving genus *Halomonas* (15). The remaining six strains all clustered in the alpha branch of *Proteobacteria* (Fig. 1). Of this subgroup, five were most closely related to the genus *Sphingomonas*. One member of the α-Proteobacteria, strain M1, which is the most oligotrophic and slow growing of all the strains used in this study, is 97% similar to *Rhodopseudomonas palustris*. The character of strain M1 correlates well with its phylogenetic placement near the slow-growing genus *Bradyrhizobium*.

***tfdA* gene fragments.** *tfdA* gene fragments were successfully amplified and sequenced from 10 strains of β-Proteobacteria and 1 strain of γ-Protobacteria. None of the strains from the α-Proteobacteria gave any amplificates with these primers. These 313 contiguous nucleotides were aligned with additional *tfdA* sequences from JMP134 and from strain RASC (Fig. 2). Three distinct classes of *tfdA* gene sequences with slight variations in each class were found. Class I included fragments from JMP134, TFD39, TFD23, K712, and TFD9 that differed from each other by 2 bp at the most. Class I *tfdA* genes are probably plasmid encoded. All strains with a class I *tfdA* gene examined so far contained broad-host-range, self-transmissible plasmids containing 2,4-D genes (2, 3, 11, 17). All of the strains with a class I *tfdA* gene were able to transfer the 2,4-D phenotype in the mating studies reported above. The class II *tfdA* sequences included identical fragments amplified from RASC, TFD6, and TFD2 which were 76% similar to those in class I. Class III included identical fragments from strains TFD31, B6-9, and I-18 which were 77% similar to class I genes and 80% similar to class II genes. Both class II and III *tfdA* genes differed from each other and from class I genes in the same nine sites corresponding to the third base pair of the codons. The *tfdA* phylogenetic tree is a simple one, with three distinct branches that are incongruent with the SSU rDNA-derived phylogeny (Fig. 3). Class I *tfdA* sequences were found in *Burkholderia*-like strains, in strains related to the *Comamonas-Rhodoferax* group, and in the *Ralstonia-Acaligenes* group, all in the β-Proteobacteria. Class II sequences are less widely distributed, found only in *Burkholderia*-like branches. However,

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[                10        20        30        40        50]
[                .         .         .         .         .]
JMP134     CATCACCAGATCTCCCACGTTCCAGCGATGCCGGTACACGAATTCCCGCT    [50]
TFD39      ..................................................    [50]
TFD23      ..................................................    [50]
B69        ......G.......G..T.......T......A.....A..C.T...T.    [50]
RASC       ......A.....C..G..T.......T......A..G..A..C.T.G.T.    [50]

[                60        70        80        90        100]
[                .         .         .         .         .]
JMP134     GTGTCGCGTGCTCGAGAAGCTCCGCAAGCAGCATCCGGCCTTCGGCCACC    [100]
TFD39      ..................................................    [100]
TFD23      ..................................................    [100]
B69        .C.....................A..C........G..A........G..G    [100]
RASC       .C.....A..............T...........G..A........G..G    [100]

[                110       120       130       140       150]
[                .         .         .         .         .]
JMP134     GGAAGGCCTTCGACGTGGCTCGCGTGCGCGCCGATGAAGAGAAACTTGCG    [150]
TFD39      ..................................................    [150]
TFD23      ..................C...............................    [150]
B69        ...C....C....T...A.C...A..A..C.....A..A...........    [150]
RASC       ...C.........T...A..A..A..A..C.....A..A...........    [150]

[                160       170       180       190       200]
[                .         .         .         .         .]
JMP134     CCCGGAGCCGGCGTGGGTTCGAACCAGCGGCCAGTTGACCGGCGGCATGG    [200]
TFD39      ...............................A..................    [200]
TFD23      ...............................A..................    [200]
B69        T..A..T..C........G..G.....T.....AC.C.....A.....C.    [200]
RASC       T..A..T..C........G..G.T..........C.C.....A.....C.    [200]

[                210       220       230       240       250]
[                .         .         .         .         .]
JMP134     CATTGCGTTGCGCTTCCGAATAGTCGGTGTCGCCGAGCAGGAAGCGGGAG    [250]
TFD39      ..................................................    [250]
TFD23      ..................................................    [250]
B69        ..........ACT...A........C..A.....A..A.T.........A    [250]
RASC       ..........ACT...A.....C..T........A..A.TA........A    [250]

[                260       270       280       290       300]
[                .         .         .         .         .]
JMP134     TTCAGTGCGTAGTGCTCGGCACGCAGCCCTTCCAACTCGGATTGGAGGTC    [300]
TFD39      ..................................................    [300]
TFD23      ...................................T..............    [300]
B69        .....C...........T..G....AT..C.G..GT..CTTC.T..AA..    [300]
RASC       .G...C...........T.......AT..C.G..GT..CTTC.T..AA..    [300]

[                310 ]
[                .   ]
JMP134     CCGAGGCAGCGCG    [313]
TFD39      .............    [313]
TFD23      .............    [313]
B69        .TCG..G..AT..    [313]
RASC       .TCG..A..AT..    [313]
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FIG. 2. Alignment of 313 nucleotides of internal fragments of *tfdA* genes from representative strains. Nucleotides identical to *tfdA* from pJP4 are represented by periods.

even in this subgroup, this *tfdA* variant is found in strains that differ by 7% at the SSU rDNA level (RASC and TFD2). However, the class III sequences were most interesting, being found both in the *Comamonas-Rhodoferax* group and in a strain of the γ-Proteobacteria, I-18, strains that differ by 24% at the SSU rDNA level. Class III genes have since been found in a collection of randomly isolated non-2,4-D degraders, including gram-positive bacilli, as well as in various gram-negative bacteria, even though the gene is not expressed (10).

An interesting result was the detection of two different *tfdA* gene variants in sibling strains. TFD23 and TFD31 are identical at the ribosomal gene level, but one harbors a class I gene and the other harbors a class III gene. Similarly, TFD6 and

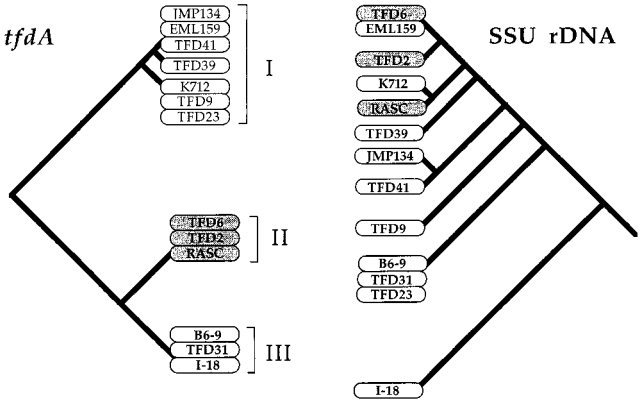

FIG. 3. Phylogenetic incongruency of *tfdA* genes and SSU rDNA from diverse 2,4-D-degrading bacteria. Dendrograms for *tfdA* and SSU rDNA are indicated. Shading indicates the type of *tfdA* sequence, either class I, II, or III. Note that branch lengths are not drawn to scale.

EML159 are rRNA siblings that carry a class II and class I gene, respectively.

None of the α-Proteobacteria yielded a PCR product when amplified with the conserved *tfdA* primers. This finding complements our observation that none of these bacteria hybridized to the *tfdA* gene, even under conditions of low stringency, indicating that any *tfdA*-like genes in the α-Proteobacteria are likely to be more divergent from the ones sequenced here (7, 11). In addition, none of the *Sphingomonas* strains in the study hybridized with a whole pJP4 probe, and similarly, no *Sphingomonas* strains scored positive for transfer of 2,4-D-degrading ability to recipient *B. cepacia* D5. Together these results suggest a reduced gene flow between members of the α- and β- or γ-Proteobacteria or poor gene expression of β- or γ-derived genes by α-Proteobacteria. Although plasmid pJP4 is a broad-host-range plasmid and has been known to transfer to α-Proteobacteria such as *Rhizobium* and *Agrobacterium* species and to γ-Proteobacteria such as *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa*, the 2,4-D pathway is not expressed in these strains of the α- or γ-Proteobacteria (3). Phylogenetically limited expression of plasmid-borne 3-chlorobenzoate-degradative genes has also been noted for the pseudomonads (8). Subsequent studies have found divergent but related sequences for the *tfdB* and *tfdC* genes in 2,4-D-degrading *Sphingomonas* strains (7, 12, 24).

With the exceptions of the minor differences within the class I pJP4-like *tfdA* sequences, there were no intermediate *tfdA* sequences. The most likely explanation of this is that the rate of horizontal transfer of the *tfd* genes is high relative to the rate at which mutations can accumulate. Examination of sequences of *tfdA* genes from a greater variety of organisms may turn up more intermediate variation.

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